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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re PATENT APPLICATION of:

HORI, ET AL. Atty. Ref: 506.35379CC2

Serial No. : 09/544,632 Group Art Unit: 1615

Filed: April 6, 2000 EXAMINER: Gollamudi S. Kishore, Ph. D

For: LIPID METABOLISM IMPROVING AGENT

DECLARATION PURSUANT TO 37 C.F.R. 1.132

Sir:

I, Goro Hori, of Tsuchiura-Shi,Ibaraki 300-1174, Japan hereby declare as follows,

I graduated from Department of Food Science and Technology, Faculty of Agriculture, Kyoto University in March, 1989. In April 1989, I have been employed by Kyowa Hakko Kogyo Co., Ltd., the assignee of the above identified application. I have since then been engaged in research and development of functional food ingredients such as cholesterol-lowering materials during the period of April 1989 to December 1999.

I am one of the co-inventors of the invention described and claimed in the application and have full knowledge of the present invention and cited references.

I have conducted the following experiment to examine the effects of bound phospholipid in protein/phospholipid complexes in improving cholesterol metabolism.

Experiment

[Materials and methods]

Preparation of protein/phospholipid complexes

To 1 kg of Promic P (isolated soybean protein, Kyowa Hakko Kogyo Co., Ltd.) was added 10 L of a 2.5% solution prepared by dispersing Elmizer AC (enzymatically modified lecithin, Kyowa Hakko Kogyo Co., Ltd.) in water. The mixture was stirred rapidly (10,000 r.p.m.) at room temperature for 10 minutes. The resulting reaction mixture was freeze-dried and then pulverized to obtain 1.1 kg of a protein/phospholipid complex (bound phospholipid content: 20 %).

The same procedures as described above was repeated, except for the concentration of Elmizer AC, whereby about 1.1 kg of protein/phospholipid complexes (bound phospholipid content: 10-50%) were obtained.

Cholesterol absorption in caco-2 cells in vitro

Caco-2 cells were provided by American Type Culture Collection (VA, USA). . In recent studies, monolayers of Caco-2 cell cultures that had been isolated from a colon carcinoma have been used as a model system in order to examine cholesterol metabolism.

The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 1% nonessential amino acids mixture. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. The monolayers became confluent 3-4 the day after seeding at

between 7 $\times 10^5$ cells and 1.2 $\times 10^6$ cells per 100-mm diameter dish; the cells were passaged at a split ratio of 4-8 by tripsinizing with 0.25% trypsin and 0.8mmol/L EDTA in PBS. Monolayers were grown in 48-well plastic dishes containing 500μ L FBS supplemented with DMEM; fresh medium was added every 2nd day. We used cultures 12-15th day after plating and were performed in medium-199/Eagle's containing 1 mmol/L HEPES.

[^{14}C]-labeled micellar cholesterol uptake in Caco-2 cells was measured by the method as follows: The final concentration of each [^{14}C]-labeled micellar solution (0.5 mL) was as follows: 3.7kBq [^{14}C]-cholesterol, 0.1mmol/L cholesterol, 1mmol/L oleic acid, 0.5mmol/L monoolein, 6.6mmol/L sodium taurocholate, 0.6mmol/Lphosphatidylcholine, and the protein/phospholipid complexes described above. The micellar solution was mixed by ultrasonic vibration.

After 14th day, the cells were rinsed two times with 1ml of PBS. A [^{14}C]-labeled micellar solution (0.5mL) containing the protein-phospholipid complexes was then added to the dishes, which were incubated at 37°C for 20 min in a CO_2 incubator. After this incubation, the cells were rinsed two times with 1mL of PBS. The cells were finally lysed in 0.1% SDS solution; then 7.5mL of Aquqsol-2 (NEN) was added, and the radioactivity in the cellular debris was counted to determine the amount of cholesterol associated with the cells.

IC_{50} means the amount of each protein/phospholipid complex to inhibit 50% of the uptake of [^{14}C]-labeled cholesterol into Caco-2 cells.

[Results]

The results are shown in Figure 1.

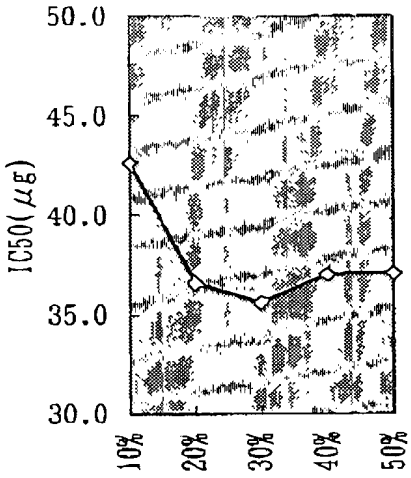

Figure 1. IC_{50} of protein/phospholipid complexes.

[Conclusion]

As shown in Figure 1, the protein/phospholipid complex containing 20-50% bound phospholipid demonstrated the maximum IC_{50} level; that is, to bring out the maximum performance of protein/phospholipid complex for improving cholesterol metabolism, the amount of bound phospholipid should be in the range from 20 to 50 wt%.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: This 1 st day of March, 2002.

Goro Hori

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